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This project tests the hypothesis that Int6 regulates the 26s proteasome and acts as a tumor suppressor for breast cancer. With the support of this grant, we were able to complete a number of studies in a genetic model system (yeast), thus firmly establishing the fact that Int6 regulates the proteasome. To further our studies in mammalian cells, the key experiment proposed in this project period is to create cell lines in which Int6 can be inactivated. The most direct and powerful way of doing this is to use RNAi to knock-down INT6 expression. We have started testing several oligos but so far have not yet successfully knocked down INT6 expression. To properly detect INT6 expression, we have obtained two antibodies against Int6 from colleagues. In the mean time, we have made anti peptide antibody against Int6 and are further improving its specificity for immunoblots. As an alternative approach to inactivate Int6 activities, we are constructing cell lines that express C-terminally truncated Int6, which may be dominant negative. We are performing RT-PCR now to screen a number of clones that may stably express these proteins.

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	6
Conclusions.....	6
References.....	6
Appendices.....	7

Figure 1-3.

INTRODUCTION

The *INT6* gene was first isolated in a screen using the mouse mammary tumor virus as an insertional mutagen to seek genes that are important for breast cancer formation. This proposal tests a hypothesis that Int6 regulates the function of the 26S proteasome, which functions to degrade growth regulatory proteins in an ubiquitin-dependent fashion. We envision that when INT6 functions are inactivated, the proteasome may be weakened to lead to accumulation of regulatory proteins, some of which are key players in the control of cell cycle and chromosome segregation. Deregulation of the cell cycle and chromosome stability are powerful factors in tumorigenesis. Our specific aims are to investigate whether altering Int6 function can influence (1) proteasome functioning and (2) chromosome segregation in human mammary epithelial cells.

REPORT BODY

Task 1: Generate cell lines with reduced *INT6* levels

a. Int6 antibody production

Two published antisera were obtained from colleagues (Desbois et al., 1996; Rasmussen et al., 2001), and used in pilot experiments to examine protein expression levels. We have started making our own by asking a company to design the peptide for generating rabbit antibodies. Figure 1 shows the results of our ongoing experiment examining the quality of a test bleed that we just received. While our current antibody is not yet as specific as we would like, it does recognize a HA-tagged Int6 when over expressed in a *yin6* null yeast strain. We expect the quality of the antibody to improve as the antibody titer increases after further boosting. We will also test the antibody using lysates prepared from human cells containing overexpressed MYC-Int6 (see below). Once we thoroughly tested the quality of the final antibody, we will decide whether it is necessary to affinity purify it.

b. and c. Test candidate DNA sequences for *INT6* knock-down

We have synthesized 3 different dsRNAs against different regions of the human *INT6* coding sequence, and tested them for their ability to suppress endogenous *INT6* expression in HeLa cells, when transfected by lipofection. The cell lysates were then analyzed using Int6 antibodies from our colleagues. At first glance, it appears that our dsRNAs did not knock down Int6 expression (Figure 2); however, there are reasons to speculate that the antibodies are picking up a nonspecific band in Western blots. We are therefore adjusting our strategy as follows:

- 1) Since we are constrained by the availability and quality of exogenous antibodies to Int6, we have started to generate our own antisera (see above). It is possible that the currently available antiserum crossreacts with a distinct protein of similar molecular weight as the endogenous wt Int6 protein. We have also built an expression vector that will express a MYC-tagged Int6 protein (see below) so we can use highly specific antibody to monitor the effectiveness of RNAi.

- 2) We have started to clone additional oligonucleotides downstream of a polIII promoter, that will drive transcription of small hairpin RNAs (shRNA). These will be expressed endogenously in transfected cells (as opposed to RNA transfection).

Alternative approach for inactivating Int6

In case RNAi does not work, we have proposed an alternative approach to inactivate Int6 functions in Task 1 by expressing a C-terminally truncated Int6 protein, Int6 Δ C. This is designed to mimic the expected protein produced as a result of MMTV insertion into the *INT6* locus (Marchetti et al., 1995). Both the wt and mutant Int6 proteins have been tagged with a N-terminal MYC-tag.

We have successfully generated stable pools of 3T3 as well as MCF10A (human mammary epithelial, Figure 3) cells infected with these retroviruses. Whereas expression of the tagged wt protein was easily detectable in these pools, we have consistently failed to express detectable levels of Int6 Δ C (e.g., Int6-1139). While this has created a technical hurdle, this result is perhaps not unexpected given the experience of others in the field (Mayeur and Hershey, 2002; Rasmussen et al., 2001). To more efficiently isolate clones that express Int6 Δ C, we are taking the following measures:

- 1) We are starting to use RT-PCR as a more sensitive assay (as compared to immunoblotting) for detecting expression of Int6 Δ C. The use of primers specific to the MYC-tag will allow specific detection of the ectopic protein as compared to the endogenous one. By comparing mRNA levels for the exogenously transfected wt and mutant proteins, we will be able to determine:

- a. Which clones are positive for Int6 Δ C; positive and negative clones will then be compared in terms of anchorage independence and other phenotypic parameters (e.g. proteasome functions), even if our detection methods lack the sensitivity to detect the protein.

- b. Whether the difference in expression between wt and mutant transfected protein occurs at the mRNA or protein levels.

- 2) To avoid the possibility of selection against Int6 Δ C in pools of infected MCF10A cells, we have also isolated 12 individual clones. These clones will also be analyzed by RT-PCR, as outlined above.

Task 3: Analyze genetic stability in engineered cells

a. Pilot experiments:

We have set up the culture system for normal human mammary epithelial cells (HMECs). Moreover, we have successfully performed pilot experiments to test the feasibility of the assay that will be needed. Initial efforts using 293 cells as the recipients for transfection were unsatisfactory, due to the strong tendency of these cells to lift off the substrate during the processing steps of the immunostaining procedure. The small cytoplasm of these cells also complicates visualization of the microtubule cytoskeleton and spindle. Both problems were solved by switching to HeLa cells. In these cells, we observed robust staining, revealing the characteristic pattern of the interphase microtubule network, and were able to detect the mitotic spindle with exquisite resolution. The high quality of the stain makes us confident that we will be able to use these assays effectively as soon as we are able to suppress endogenous Int6 expression.

KEY RESEARCH ACCOMPLISHMENTS:

1. We have obtained polyclonal anti-Int6 antibody (Figure 1).
2. We have created stable cell lines that express MYC-tagged Int6. This will facilitate the screen for effective RNAi (Figure 2).
3. We have also created cell lines that may express Int6 Δ C, which may be dominant negative to interfere with endogenous Int6 functions (Figure 3).

REPORTABLE OUTCOMES:

Meeting Abstracts:

1. "INT6 IS A POTENTIAL BREAST TUMOR SUPPRESSOR GENE, " Yen HS, Nelson L, Sha Z, Mercado I, and Chang EC. 2003. Breast Cancer Center Retreat, Baylor College of Med. and the Methodist Hospital.
2. "A possible molecular mechanism of action for the potential tumor suppressor gene INT6 provided by studies in fission yeast." Yen H.-c S., Ren G, Nelson, L., Sha, Z., Sap J, and Chang E. 2003. San Antonio Breast Cancer Symposium.
3. "Int6 is a potential breast tumor suppressor that regulates the 26S proteasome" Yen H.-c S., Ren G, Nelson, L., Sha, Z., Sap J, and Chang E. 2003. ASCB meeting. (Mol. Biol. Cell 14: 166a, 2003).

CONCLUSION:

This project tests the hypothesis that Int6 regulates the 26s proteasome and acts as a tumor suppressor for breast cancer. With the support of this grant, we were able to complete a number of studies in a genetic model system (yeast), thus firmly establishing the fact that Int6 regulates the proteasome. To further our studies in mammalian cells, the key experiment proposed in this project period is to create mammalian cell lines in which Int6 can be inactivated. The most direct and powerful way of doing this is to use RNAi to knock-down *INT6* expression. We have started testing several oligos but so far have not yet successfully knocked down *INT6* expression. To properly detect *INT6* expression, we have obtained two antibodies against Int6 from colleagues. In the mean time, we have made anti peptide antibody against Int6 and are further improving its specificity for immunoblots. As an alternative approach to inactivate Int6 activities, we are constructing cell lines that express C-terminally truncated Int6, which may be dominant negative. We are performing RT-PCR now to screen a number of clones that may stably express these proteins.

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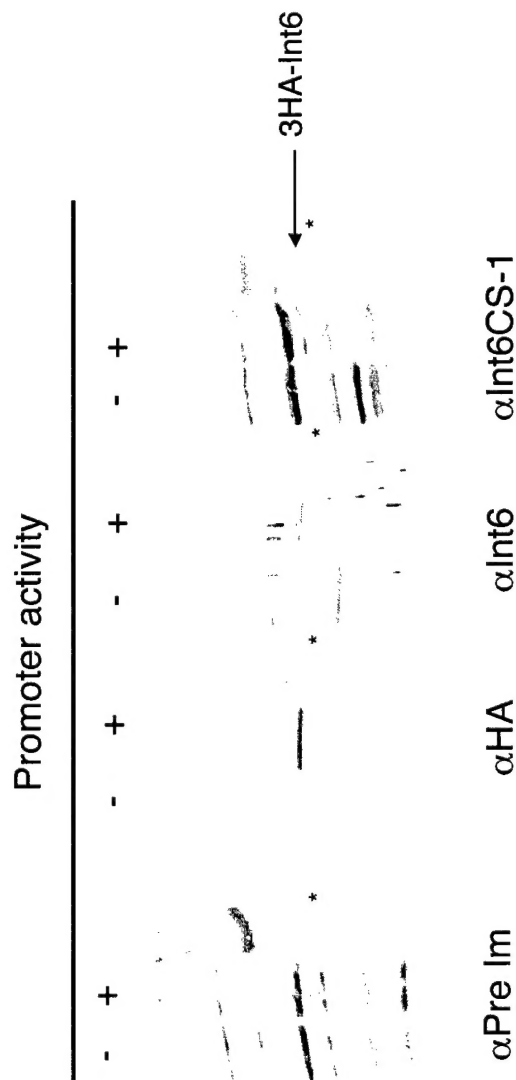


Figure 1. Detection of HA-Int6 protein using various antibodies. *ynf6* cells were transformed with a vector expressing triple HA tagged Int6, 3HA-Int6, whose expression was controlled by the thiamine repressible *nmt1* promoter. "-" indicates that the *nmt1* promoter was repressed; while "+" indicates that *nmt1* was fully active. The same cell lysate was analyzed by Western blots using three antibodies, as marked at the bottom of the blot. α -Int6 is a control polyclonal Int6 antibody (Desbois et al., 1996). We named our antibody, Int6CS-1 and the pre immune control serum is named "Pre Im." Although this antibody showed a high background, it did recognize a band co-migrating with 3HA-Int6, which appears just above the 50 kDa MW marker band (*).

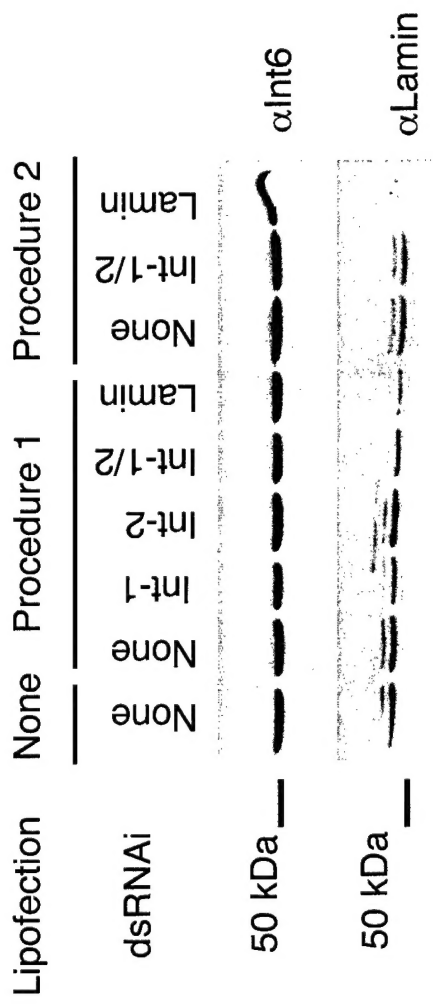


Figure 2. Testing of RNAi for knocking down *INT6* expression. Hela cells were subjected to transient lipofection using two different protocols with two synthetic double-stranded RNAs directed against human *INT6*, or with a previously validated RNAi control against human Lamin A/C. Equal amounts of total lysate were analyzed by Western blots using antibodies against Int6 (Desbois et al., 1996) or lamin A/C.

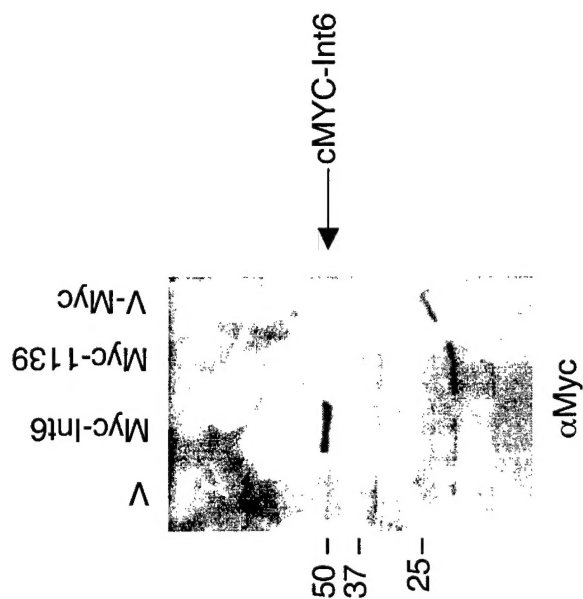


Figure 3. Expression of full length and truncated Int6 in Human MCF 10A breast epithelial cells. MCF 10A cells were infected with different stocks of VSV-G-pseudotyped recombinant retrovirus. V: vector control (pBabe-puro); Myc-Int6: pBabe-puro expressing Myc tagged full length Int6; Myc-1139: pBabe-puro expressing C-terminally truncated Int6; V-Myc, pBabe-puro expressing just the Myc epitope control. Pools of puromycin-resistant cells were selected and equal amounts of lysate subjected to immunoblotting with anti-Myc. The Myc tagged truncated Int6 (1139) is expected to run as a 25 kDa protein.